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(57) Abstract

This invention relates to a method of producing peracetyloxazolines from peracetyl saccharides. The method involves reacting the starting material, a peracetyl saccharide, with a reagent combination, to directly produce the peracetyl oxazoline. This method may be used for the activation of oligosaccharides, wherein an oligosaccharide containing a reducing GlcNAc terminus is activated by the formation of an oxazoline at the terminal GlcNAc, and then coupled with a bifunctional spacer to provide an oligosaccharide-spacer conjugate. The activated oligosaccharide-spacer conjugate is then coupled to a protein, such as granulocyte colony stimulating factor or γ-interferon, providing a neoglycoprotein conjugate. The invention provides a method for forming neoglycoprotein conjugates which may improve biological and physiochemical properties of the protein. For example, serum lifetime or efficiency of drug delivery of the peptide to a target organ or cell may be improved.

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OLIGOSACCHARIDE OXAZOLINES, OLIGOSACCHARIDE CONJUGATES AND METHODS OF PREPARATION THEREOF

5

Technical Field

This invention relates to methods for the preparation of oligosaccharide oxazolines and to oligosaccharide
10 conjugates and the preparation thereof.

Background Art

Glycoproteins, covalent conjugates of carbohydrates and proteins, are essential in maintaining and regulating intracellular and extracellular biological activities in a
15 living organism. The external surface of the cellular plasma membrane is enriched with glycoproteins which function as cell surface receptors for growth factors, hormones and toxins. In addition, glycoproteins secreted into extracellular fluids exist as growth factors, hormones,
20 enzymes and antibodies. The effect of the carbohydrate moieties on the biological activity of glycoproteins is a subject of continuing studies. It has been shown that carbohydrate moieties play a vital role in regulating the structure and function of a glycoprotein. The carbohydrate
25 moieties may mediate the cellular uptake of the protein, protect the protein from denaturation and proteolytic degradation, or modulate physical properties of the protein such as viscosity, stability, circulatory lifetime and binding capacity (Experientia (1982) 38, pp. 1129-1162; and
30 Ann. Rev. Biochem. (1988) 57, pp. 785-838). Recent studies also demonstrated the ability of the oligosaccharide moieties of glycoproteins to reduce self-association of the proteins (Biochem. (1990) 29, pp. 2482-2487).

Glycosylation is a biologically important modification
35 of the covalent structure of a protein. There are two categories of glycosylation recognized in most eukaryotic systems: O-linked glycans are attached to proteins via an α-glycosidic linkage to either serine or threonine, while the more frequent N-linked glycans are covalently attached

via a β-glycosidic bond to an asparagine moiety. Protein N-glycosylation occurs co-translationally in the endoplasmic reticulum with the oligosaccharide $Glc_3Man_9GlcNAc_2$ being transferred en bloc from the lipid carrier dolichol
5 diphosphate to an asparagine residue contained in the peptide sequence of Asn-X-Ser(Thr). Subsequent processing of the nascent glycoprotein in the Golgi by a battery of glycosidases and glycosyltransferases gives rise to a myriad of possible structures for the mature N-linked glycan. Many
10 literature reviews of the structure, function and biosynthesis of N-linked glycans have been recently published (Baenzinger in The Plasma Proteins (1984) IV pp. 271-315; Snider in Biology of Carbohydrates (1984) 2, pp. 164-198; and Kornfeld and Kornfeld in Ann. Rev. Biochem.
15 (1985) 54 pp. 631-664).

A necessary step in obtaining information concerning N-glycan structure, function and biosynthesis is the development of synthetic methodology for the preparation of specific asparagine N-linked glycans, as well as the
20 oligosaccharide dolichol diphosphate lipid intermediate and other carbohydrate derivatives.

Synthesis of all these glycosides is dependent on the chemical "activation" of the terminal GlcNAc moiety of the glycan. One successful glycosyl activation strategy
25 involves the acid-catalyzed formation of an oxazoline intermediate from peracetylated oligosaccharides. For instance, Warren et al. in International Patent Application PCT/US87/01832 describe the synthesis of a high-mannose oligosaccharide asparagine derivative, from an oxazoline
30 derivative and Warren et al. (Carbohydr. Res. (1984) 126 pp. 61-80) also describe the use of an oxazoline to prepare the dolichol diphosphate lipid intermediate.

The synthetic methodology utilized by Warren et al. in the preparation of asparagine-linked high-mannose
35 oligosaccharide derivatives was based on a three-step protocol: (1) addition of azide to the oxazoline intermediate so as to obtain the β-linked azide derivative,

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(2) reduction of the β-azide to a β-1-amino glycoside, and
(3) coupling of the glycosylamine to the appropriately
protected aspartate moiety. The general synthesis of Asn-
linked oligosaccharides is thus ultimately dependent on a
5 mild and efficient method for oxazoline generation. The
Asn-linked oligosaccharides are of importance because they
may be incorporated into N-linked glycopeptides by
condensation of various peptides to the glycosylated
asparagine amino-acid moiety. These N-linked glycopeptides
10 have potential use, not only as glycosidase substrate
analogues, but also as model systems for studying the
structure and dynamics of N-linked glycoproteins.

Peracetyl oxazolines of mono- or disaccharides are
traditionally prepared via chloroacetolysis (acetyl chloride
15 in concentrated HCl) of the corresponding peracetyl
saccharide. Warren et al. (Carbohydr. Res. (1977) 53 pp.
67-84, (1980) 92 pp. 85-101, and (1984) 126 pp. 61-80)
describe the preparation of the peracetylated oxazolines of
various saccharides. A severe problem associated with the
20 chloroacetolysis procedure is the susceptibility for
cleavage of any acid-labile glycosidic bonds. For instance
any α-D (1→6) glycosidic linkages in the particular
oligosaccharide and the β-D (1→4) linkages between the two
N-acetylglucosamines (GlcNAc) of the oligosaccharide's
25 chitobiose core are particularly prone to acid-catalyzed
glycosidic cleavage. The relatively harsh conditions of the
chloroacetolysis reaction are therefore not amenable to many
structurally complex oligosaccharides.

The direct formation of a peracetyl oxazoline from the
30 peracetate using a Lewis acid as catalyst is an alternative
approach to the chloroacetolysis method. Matta et al. in
Carbohydr. Res. (1973) 26 pp. 215-218, describe the
formation of an oxazoline from the β-D-anomer of peracetyl
saccharides using anhydrous ferric chloride. Ferric
35 chloride does not, however, catalyze formation of the
oxazoline from the predominant α-D-anomer of the peracetyl
saccharide. Because of its inability to promote oxazoline

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formation from the major anomer the ferric chloride method
is of limited applicability.

   Scrivastava describes a method, using ferric chloride,
for oxazoline generation from the α-D-anomer of peracetyl
5 glucosamine (Carbohydr. Res. (1982) 103 pp. 286-292). When
this method was applied to peracetyl oligosaccharides the
predominance of side reactions led to low yields of desired
product. Nakabayashi et al., in Carbohydr. Res., (1986) 150
C7-C10 and Warren et al., in International Patent
10 Application PCT/US87/01832 describe an alternative Lewis-
Acid catalyzed procedure for oxazoline generation. This
method is designed to circumvent the two aforementioned
problems of poor anomeric selectivity and low overall yields
of oxazoline. Using one equivalent of the Lewis acid
15 trimethylsilyl trifluoromethanesulfonate these authors
formed a peracetyl oxazoline from a heptasaccharide
peracetate isomer (Man$_5$GlcNAc$_2$). Nakabayashi et al. claimed
that the heptasaccharide oxazoline was accessible from
either the α or β anomer, and they also reported that no
20 glycosidic bond cleavage was detected.

   Activated oligosaccharide derivatives may be reacted
with proteins to form oligosaccharide-protein conjugates
(neoglycoproteins). Because oligosaccharides have a wide
spectrum of biological activity, the formation of
25 neoglycoproteins is valuable for improving the biological
and physiochemical activity of proteins. Marburg et al.
reported a method of preparing oligosaccharide conjugates
wherein oligosaccharides are activated by the addition of
carbonyldiimidazole or nitrophenyl chloroformate and then
30 reacted with a spacer group with a pendant electrophilic
group followed by reaction with a nucleophilic group on a
protein (U.S. Pat. No. 4,830,852 (1989)). Some
neoglycoproteins have improved thermal stability and
increased stability towards proteases and denaturants. For
35 example, after incubation at 60°C for 10 min, lactosylated
E. coli L-asparaginase retains 63% of its activity, whereas
the native enzyme retains only 19%. The same modification

is also reported to stabilize the asparaginase towards
proteolytic digestion. Lactosylated asparaginase retains
50% of its activity after a 60 min incubation with
subtilisin, whereas native asparaginase is completely
5 deactivated after only 20 min incubation (J. Biol. Chem
(1977) 252 pp. 7678). In addition, neoglycoproteins often
have longer circulatory lifetimes. For example, dextran-
amylase conjugates have a circulatory lifetime which is 4.5
times longer than the unmodified enzyme (Trends Biochem.
10 Sci. (1978) 3 pp. 79).

     Another interesting aspect of neoglycoproteins is their
potential in efficient drug delivery, especially in
targeting therapeutic proteins to particular cells or
organelles. Endocytosis of glycoproteins is often mediated
15 by the interaction of cell-surface receptors and the
glycoprotein carbohydrate components. The attachment of the
appropriate carbohydrate components could result in the
delivery of neoglycoproteins to specific tissues or organs
of the body. For example glycoproteins with a terminal
20 galactose are selectively removed from circulation by
hepatocytes (Adv. Enzymol. Relat. Areas Mol. Biol. (1978) 41
pp. 99 - 128). In another example, by attaching lactose to
the bovine pancreatic RNAse A dimer, the uptake of the
protein was shifted from the kidney to the liver, where
25 galactosyl receptors are located (J. Biol. Chem. (1978) 253,
pp. 2070-2072). It has also been shown that the binding
affinity of mannosylated bovine serum albumin to the
mannose-receptors on rabbit alveolar macrophages increases
dramatically, even with a small increase in the number of
30 mannose attached (Biochemistry, (1988) 15 pp. 3956). Thus,
neoglycoproteins hold great therapeutic potential,
especially in the treatment of diseases caused by enzyme
deficiencies.

     There have been numerous methods reported in the
35 literature for the covalent coupling of carbohydrates to
proteins (Advances in Carbohydrate Chemistry and
Biochemistry (1980) 37 pp. 225-281; CRC Critical Reviews in

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Biochemistry (1981) pp. 259-306; Glycoconjugates (1982) IV
pp. 57-83). For example, p-aminophenyl glycosides of the
oligosaccharide may be diazotized and allowed to react with
lysyl, tyrosyl and histidyl residues of the protein.

5 Alternatively, the p-aminophenyl glycoside may be converted
to an isothiocyanate and attached to amino functional groups
on the protein. Another method of neoglycoprotein
production involves amidation between a carboxyl group and
an amine facilitated by dicyclohexylcarbodiimide (DCC), 3-

10 (3-dimethylaminopropyl)-1-ethylcarbodiimide (DAEC) or mixed
anhydrides. The carboxy component may be the protein's
side-chain carboxy groups or aldonic acids generated by
oxidation of the terminal oligosaccharide residue. The
amino component may be aminoglycosides or lysines of the

15 protein. Hydrazido-oligosaccharide derivatives may be
converted by treatment with nitrous acid into acyl azides,
which are highly reactive towards lysine residues of
proteins.

      Direct coupling of carbohydrates to proteins may also

20 be achieved by reductive amination. In this procedure, the
reducing terminus of the oligosaccharide is allowed to react
with the amino groups of the protein to form a Schiff base;
the Schiff base is subsequently reduced with sodium
cyanoborohydride, providing a hydrolytically stable amine

25 linkage between carbohydrate and protein. In general, there
is a current need for efficient and specific methods which
allow coupling of proteins and oligosaccharides.

                    Summary of the Invention
      The present invention relates, in one embodiment, to a

30 novel method for the direct preparation of peracetyl
oxazolines from peracetyl oligosaccharides. In another
embodiment, the invention also relates to the use of
peracetyl oxazoline derivatives of oligosaccharides as
efficient glycosylation agents for the chemical synthesis of

35 glycoconjugates (i.e. O-glycosides, glycolipids,
glycopeptides, etc.). Using the methods described in the
invention, an oligosaccharide, containing a reducing GlcNAc

terminus, may be activated by the formation of an oxazoline
at the terminal GlcNAc, and then coupled to a bifunctional
spacer. In accordance with an embodiment of the invention,
the oligosaccharide may then be coupled to a therapeutically
5 useful protein, such as granulocyte colony stimulating
factor or γ-interferon, providing a neoglycoprotein
conjugate. The formation of a neoglycoprotein conjugate of
a therapeutically valuable protein may enhance serum
lifetime of the protein, or may improve the efficiency of
10 drug delivery of the protein to a target organ or cell.

Brief Description of Figures

The foregoing features of the invention will be more
readily understood by reference to the following detailed
description taken with the accompanying figures, in which:
15   Fig. 1 illustrates possible reaction intermediates in
the generation of oxazoline product from peracetyl
saccharides using reagent combinations described herein.

Figs. 2 and 3 illustrate the structure of the high
mannose type oligosaccharides, $Man_5GlcNAc$, $Man_6GlcNAc$, the
20 two isomers of $Man_7GlcNAc$, and $Man_8GlcNAc$.

Figs. 4 and 5 are a reaction scheme showing the
preparation of an oligosaccharide-protein conjugate in
accordance with the invention as described in Example 4.

Detailed Description of Specific Embodiments

25 **A.   Preparation of Oligosaccharides**

The oligosaccharide derivatives used in embodiments of
this invention are derived from any oligosaccharide
containing a 2-acetamido-2-deoxyglucopyranose (GlcNAc) at
the reducing terminus of the oligosaccharide. Suitable
30 oligosaccharides include, but are not limited to, high
mannose type oligosaccharides, hybrid-type oligosaccharides
and complex-type oligosaccharides. Preferred sources of the
oligosaccharides include oligosaccharides from glycoproteins
and glycopeptides which are enzymatically released by the
35 action of endoglycosidases such as endo-H and amidases such
as N-Glycanase; or chemically released, for example, by
hydrazinolysis. Examples of such glycoproteins include high

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mannose glycoproteins such as 7S globulin (J. Biol. Chem.
(1981) 256 pp. 7708), ribonuclease B (Hoppe Seyler's Biol.
Chem. (1986) 367 pp. 191), and ovalbumin (J. Am. Chem. Soc.
(1984) 106 pp. 2412.) The structure of high mannose type
5 oligosaccharides are illustrated in Figs. 2 and 3. Other
examples include complex-type glycoproteins such as α-1-acid
glycoprotein, transferrin and fetuin (Anal. Bioch. (1987)
162 pp. 485), as well as hen ovomucoid (J. Biol. Chem.
(1982) 257 pp. 12809) and turkey ovomucoid (J. Biol. Chem.
10 (1983) 258 pp. 3099). The complex-type oligosaccharides may
or may not be sialylated. However, in the chemical
modification step, the desialylated complex oligosaccharide
is preferred. The terminal sialic acid may be added in a
later step using sialyl transferase.
15 B.   Preparation of Oligosaccharide Oxazolines
        Attempts by scientists at Genzyme Corporation to form
an oxazoline from a high-mannose nonasaccharide peracetate
(Man$_8$GlcNAc) using the Nakabayashi procedure (op. cit.) met
with only marginal success. While the minor β-D-anomer (ca
20 10%) rapidly formed the desired oxazoline, the predominant
α-D-anomer was extremely sluggish in forming product using
the Nakabayashi procedure. Prolonged reaction times and
elevated temperatures, while giving increased yields of
oxazoline also led to significant glycosidic bond cleavage,
25 severely compromising the purity of the final product.
These unsatisfactory results with the nonasaccharide
peracetate have prompted the development of an alternative
method for oxazoline preparation. The embodiment described
below of the present invention is better suited for the
30 preparation of peracetyl oxazolines from structurally
complex and acid-labile oligosaccharide peracetates.
        In general, the invention features, in one embodiment,
a method for the preparation of oxazolines from peracetyl
saccharides containing an N-acetylglucosaminyl residue as
35 the reducing terminus of the saccharide. The conditions
utilized in this method are sufficient to catalyze formation
of the oxazoline from either the α or β anomer of the

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peracetyl saccharide, yet mild enough so that no
concommitant glycosidic bond cleavage is detected. The
method involves reacting a peracetyl saccharide with a
particular combination of reactive compounds capable of
5   catalyzing the formation of an intermediate acetoxonium ion
so as to subsequently produce the peracetyl oxazoline. The
critical aspect of this embodiment is the judicious
combination of two reagents, which when used in tandem,
catalyze oxazoline formation. Treatment of the starting
10 peracetyl saccharide with either reagent alone, however,
results in no reaction. Neither reagent, when alone or when
combined with the other reagent, promotes any deleterious
side-reactions of the starting peracetyl saccharide. This
contrasts to the behavior of the more reactive Lewis acid,
15 trifluoromethanesulfonic acid, which while leading to
oxazoline formation, gives significant glycosidic cleavage
when particular oligosaccharides are utilized.

In accordance with an embodiment of this invention, a
peracetyl oxazoline may be prepared from a peracetyl
20 saccharide which may be derived from either a naturally
occurring or a synthetic mono-, di-, or oligosaccharide
containing an N-acetyl-glucosamine at the reducing terminus
of the saccharide. The oligosaccharide obtained from
natural sources may be produced by digestion of an N-Linked
25 glycoprotein or glycopeptide, with either (1) an
endoglycosidase such as endo-H or (2) an amidase such as N-
glycanase or they may be obtained by (3) hydrazinolysis of
the N-linked glycoprotein or glycopeptide.

If a peracetyl oligosaccharide is derived from natural
30 sources it may be either a high-mannose, a complex or a
hybrid oligosaccharide. The oligosaccharide will typically
contain one to twelve residues of neutral hexoses, with at
least one residue, the reducing terminal residue, being an
N-acetylhexosamine. The oligosaccharide may also contain
35 fucose or sialic acid residues. In addition, the reducing
N-acetylglucosamine residue may be present in either the α
or the β configuration.

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While the preferred protecting group for the hydroxyl
functionalities of the oligosaccharide is the acetyl residue
other protecting groups are amenable for oxazoline
preparation. For instance pertrichloroacetyl,
5 pertrifluoroacetyl or perbenzoyl oligosaccharides may also
be used to generate reactive oligosaccharide oxazoline
derivatives. In some cases it may be preferable to prepare
oxazoline from pertrichloroacetyl or pertrifluoroacetyl
oligosaccharides as these two protecting groups are more
10 easily deprotected by base hydrolysis than the peracetyl
oligosaccharides. Perbenzoyl oligosaccharides are, by
contrast, hydrolytically more stable than their peracetate
analogs.

The peracetyl saccharide is reacted with a combination
15 of reagents capable of generating the formation of
intermediates 1, 2, or 3 (Fig. 1). The initial acetolysis
catalyzed by the reagent combination may lead to the
formation of an intermediate glycosyl halide 1, an intimate
ion-pair 2, or a glycosyl cation 3 (Fig. 1). In the process
20 of this invention, all three intermediates may undergo
neighboring-group addition of the C-2 N-acetamide group to
preferentially or exclusively yield the peracetyl oxazoline.
Glycosyl halide 1 may undergo an intramolecular S_N2 reaction
or similarly heterolysis of the C-1 halogen bond may lead to
25 the intimate ion pair 2 or the glycosyl cation 3, both of
which are stabilized immediately by intramolecular attack of
the C-2 acetamide group. In each case, the final product is
peracetyl oxazoline. Reagent combinations capable of
generating the desired peracetyl oxazoline include, but are
30 not limited to, mixtures of boron trifluoride-etherate (BF_3-
OEt_2) and bromotrimethylsilane (TMS-Br), chlorotrimethyl-
silane (TMS-Cl), or iodotrimethylsilane (TMS-I). The
combination of BF_3-OEt_2 and TMS-Br is preferred. One fifth
equivalent of the peracetyl saccharide is reacted with a
35 ratio of one equivalent of BF_3-OEt_2 to one to ten equivalents
of TMS-Cl or TMS-Br, with a ratio of one equivalent of BF_3-
OEt_2 to one equivalent of TMS-Cl or TMS-Br being preferred.

In addition, from one to ten equivalents of an acid
scavenger per equivalent of BF_3-OEt_2 may be added to the
reaction mixture so as to accelerate oxazoline product
formation and protect against any acid-catalyzed

5   decomposition of the oxazoline product. For example 2,4,6-
collidine, pyridine, triethylamine, or other tertiary amines
may be used as the acid scavenger, with 2,4,6-collidine
being preferred.

    The foregoing method is carried out in an anhydrous

10 organic solvent with dichloroethane being preferred. The
reaction conditions include temperatures of 0°C to 50°C, and
times of 2 to 24 hours with a preferred temperature being
20°C and a preferred time being 2 hours. The reaction may
be monitored by thin layer chromatography (tlc) on glass

15 plates coated with analytical grade silica gel using 10:1
(v/v) chloroform-methanol or 10:1 (v/v) ethyl acetate-
methanol as the solvent system.

    After the reaction is judged to be complete, the
product perecetyl oxazoline may be isolated and purified by

20 silica gel column chromatography. In one embodiment, the
reaction mixture is washed with water, the organic layer is
dried over Na_2SO_4, concentrated, and the crude high-mannose
oligosaccharide oxazoline is purified by column
chromatography on Kieselgel 60 silica (230-400 mesh) with

25 elution effected by 40:1 chloroform-methanol. The yield is
approximately 95% based on the peracetyl saccharide.

    The peracetyl oxazolines prepared according to this
invention may be used as glycosyl donors in the chemical
synthesis of oligosaccharides, glycolipids, glycopeptides,

30 glycoproteins, and other useful β-D-glycosides. In the
presence of the proper reagent combination any of the
aforementioned intermediates (1, 2, or 3) may react to form
oxazoline. The reagent combination may be a 1:1 mixture of
bromotrimethylsilane or chlorotrimethylsiliane, and boron

35 trifluoride etherate. In addition, one equivalent of an
acid scavenger such as 2,4,6-collidine, for example, may be
used to accelerate oxazoline generation and also to prevent

acid catalyzed decomposition of both the oxazoline and the
starting saccharide. The present invention provides a
method greatly superior to the method of Nakabayashi et al.
(op. cit.) which was found to be unsuitable for the
5   generation of oxazoline from the predominant alpha anomers
of Man$_n$GlcNAc (where n is an integer in the range from and
including 5 through 8) oligosaccharides isolated from the
soybean 7S globulin. Thus when a 90:10 α:β mixture of the
peracetates of Man$_n$GlcNAc were treated with trimethylsilyl
10 trifluoromethanesulfonate according to the Nakabayashi et
al. protocol, only the minor β-isomer formed oxazoline while
the α-anomer resisted reaction. Prolonged reaction times or
elevated temperatures led to significant glycosidic bond
cleavage and generation of unacceptable amounts of side
15 products. Optimum conditions for oxazoline formation were
determined using 2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-
α-D-glucopyranose as a model study. Tables 1 and 2
summarize the results. Table 1 shows data describing the
reaction of 2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-α-D-
20 glucopyranose with various combinations of the reagents TMS-
bromide and BF$_3$ etherate. Table 2 shows data describing the
reaction of 2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-α-D-
glucopyranose with a 1:1 ratio of TMS-bromide and BF$_3$-OEt$_2$ in
the presence of various amounts of the acid scavenger 2,4,6-
25 collidine.

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TABLE 1

<u>Preparation of oxazoline from 2-acetamido-1,3,4,6-</u>
<u>tetra-O-acetyl-2-deoxy-α-D-glucopyranose using various</u>
<u>reagent combinations</u>[*].

Entry	Reagent Combination[a]	Acid Scavenger[b]	Time	Yield
1	BF_3-OEt_2	0	24 hrs	0%
2	TMSBr	0	24 hrs	0%
3	BF_3-OEt_2 + TMSBr	0	16 hrs	70%
4	BF_3-OEt_2 + TMSBr	1	24 hrs	95%

[*]50 mg of starting material was dissolved in 2 ml of
anhydrous dichloroethane and stirred with the corresponding
reagent combinations at room temperature. The reaction was
monitored by t.l.c. (10:1, v/v chloroform/methanol). When
the reaction was complete, it was washed with water, dried
with Na_2SO_4, concentrated in vacuo, applied to a column of
silica gel (Kieselgel 10; 230-400 mesh) and eluted with 40:1
(v/v) chloroform/methanol. The product was identified by
^{1}H NMR.

[a]1 molar equivalent of the reagent combination to one
molar equivalent of starting material was used.

[b]Equivalents of 2,4,6-collidine per equivalent of
starting material.

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TABLE 2

Preparation of oxazoline from 2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-α-D-glucopyranose using a 1:1 combination of TMS-bromide and BF₃-OEt₃* and various equivalents of acid scavenger.

Entry	Equivalents[a]	Acid Scavenger[b]	Time	Yield
1	1	0	24 hrs	55%
2	1	1	24 hrs	90%
3	2	2	15 hrs	97%
4	5	5	7 hrs	95%
5	10	10	5 hrs	96%

*50 mg of starting material was dissolved in 2 ml of anhydrous dichloroethane and stirred with the corresponding reagents at room temperature. The reaction was monitored by t.l.c. (10:1, v/v chloroform/methanol). When the reaction was complete, it was washed with water, dried with Na₂SO₄, concentrated in vacuo, applied to a column of silica gel (Kieselgel 10; 230-400 mesh) and eluted with 40:1 (v/v) chloroform/methanol. The product was identified by ¹H NMR.

[a]mmol of the 1:1 TMS-bromide-boron trifluoride etherate combination per mmol of starting material.

[b]Equivalents of 2,4,6-collidine per equivalents of the 1:1 combination of TMS-bromide and boron trifluoride etherate.

The present invention provides methods superior to currently available methods for preparing peracetyl oxazolines from structurally complex oligosaccharides. Due to the mild reaction conditions, peracetyl oligosaccharides
5 containing acid-labile linkages may be used in the process of this invention. Unlike most previously reported methods, the invention can work equally well with either the alpha or the beta anomer of the starting peracetyl saccharide.

C.   **Preparation of Oligosaccharide-Spacer Conjugates**

10 The next step is the acid-catalyzed addition of a bifunctional spacer to the oligosaccharide-oxazoline. The reaction yields specifically a β-glycosidic bond between the terminal GlcNAc of the oligosaccharide and the bifunctional spacer. The oligosaccharide-spacer conjugate has the
15 general formula: Os-A-B-C where Os is the oligosaccharide with a terminal GlcNAc, A is β-O- or β-NH-C=O, B is a spacer and C is either a cysteine-reactive group such as:

20
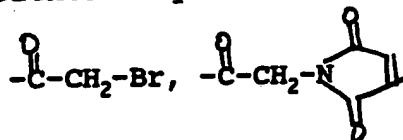
or a lysine reactive group such as:

25 The bifunctional spacer may then be represented by A'-B-C where A' is a functional group which is reactive towards the oxazoline, and B and C are as described above. There are numerous examples of bifunctional spacers reported in the literature (for example, those cited in Methods in
30 Enzymology 91, Part 1, pp. 581-609; and Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes Inc. (1989)). A preferred embodiment of the bifunctional spacer may be represented by $HO-(CH_2)_n-X-Y-(CH_2)_n-Z$ wherein:

n is an integer in the range from and including 0
35 through 10;

X is C=O, C=NH, $(CH_2)_n$, or N-R and R is H, or CH_3;

Y is C=O, C=NH, $(CH_2)_n$, or N-R, S; and

Z is N-R or $(CH_2)_n$

5   The bifunctional spacer may have the electrophilic group, for example $-\overset{O}{\underset{}{C}}-CH_2-Br$, already attached, or it may be masked by protecting groups such as $HNC(O)CF_3$, which may be deprotected and further functionalized after the addition to the oligosaccharide-oxazoline. In a typical example, N-

10   trifluoracetyl-5-amino-pentanol, a bifunctional spacer, is allowed to react with oligosaccharide-oxazoline in the presence of p-toluenesulfonic acid. The reaction proceeds at ambient temperature, under a nitrogen atmosphere for 16 hr. The O- and N- protecting groups on the resultant

15   oligosaccharide-spacer conjugate are removed by treatment with NaOMe/MeOH in ambient temp for 4-24 hr. The oligosaccharide-spacer conjugate so obtained is further functionalized with the addition of an electrophilic moiety. Suitable reagents for such transformation include agents

20   such as:
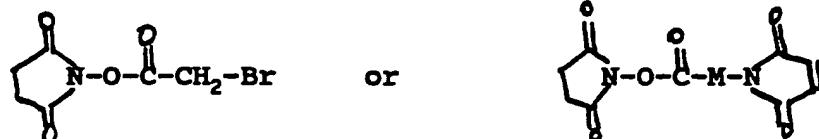
where M is an aromatic ring or $(CH_2)_n$, where n is an integer

25   from and including 1 through 5.

Alternatively the oligosaccharide-oxazoline is treated with trimethylsilyl-azide in the presence of a Lewis acid such as tin(IV) chloride. The resultant oligosaccharide-azide is subsequently reduced to give the 1-amino-

30   oligosaccharide. The reduction may be accomplished by hydride transfer, for example using lithium aluminum hydride, or by catalytic hydrogenation, for example using palladium(0) on charcoal or platinum oxide (Adams catalyst)

as catalysts. The 1-amino-oligosaccharide so obtained may
be coupled with bifunctional spacers such as:
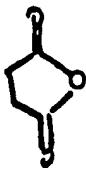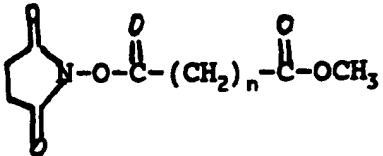
D.   Preparation of the Protein

The proteins being utilized for the synthesis of
oligosaccharide-protein conjugates using the methods of this
invention include, but are not limited to, proteins of
therapeutic value such as serum albumin, γ-interferon,
granulocyte-colony stimulating factor (G-CSF), superoxide
dismutase and interleukin-2. In addition to conjugates
formed with proteins, conjugates may be formed similarly
with peptides and polypeptides. The use of the term
"protein" in the disclosure and claims herein, in this
context, is introduced to include peptides and polypeptides.

The proteins utilized in embodiments of this invention
include those possessing nucleophilic groups such as
cysteine, lysine, or histidine. The cysteines may be
present either as a free cysteine or as a disulfide.
Preferably, these sulfhydryl groups are not required for the
biological activities of the protein being modified. These
sulfhydryl groups may be sterically or chemically
accessible, for example, Cys-34 on bovine serum albumin,
which may react readily with the activated oligosaccharide
in the conjugation step. Others may resist chemical
modification due to steric hindrance or disulfide formation.
Nucleophilic groups that are sterically inaccessible, for
example, Cys-17 on granulocyte-colony stimulating factor (G-
CSF), may be exposed for reaction by adding denaturants such
as urea or guanidine, or by adding detergents like tween-20
and sodium dodecylsulfate. Disulfides on proteins, for
example, those on murine γ-interferon, can be reductively
cleaved by reagents such as dithiothreitol, 2-

mercaptoethanol or sodium borohydride. The reductively
exposed sulfhydryls may then be reacted with the activated
oligosaccharides.

   E.   Conjugation of Protein with Activated Oligosaccharide

5   to Provide Neoglycoprotein.

      Neoglycoproteins obtained in accordance with an
embodiment of this invention may be represented by the
formula: Os-A-B-C-Nu-Pr wherein:

      Os   is the oligosaccharide with the terminal GlcNAc;

10   A   is β-O- or β-NH-C=O;

      B   is the spacer group defined in part A;

      C   is the product of the reaction between the
           electrophilic group on the oligosaccharide with
           the nucleophilic group on the protein having the

15   formula:

$$-\overset{O}{\overset{\|}{C}}-CH_2-, \quad -\overset{O}{\overset{\|}{C}}-CH_2-N\diagup \quad \text{or} \quad -\overset{O}{\overset{\|}{C}}-$$
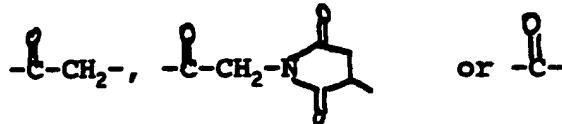
      Nu   is the nucleophilic group on the protein; and

20   Pr   is the protein.

      The protein-oligosaccharide conjugate is formed by
reacting the suitably prepared protein with the activated
oligosaccharide derivatives, preferably in molar ratios of
1:1 to 1:30 respectively, in aqueous buffered solution, at a

25 pH of 7 to 9. The reaction is usually performed under a
nitrogen atmosphere, at temperatures from 0 to 37°C, for a
time period ranging from one to twenty-four hours. Examples
of such a reaction include:

30      $Os-(C_5H_{10})-NH-\overset{O}{\overset{\|}{C}}-(CH_2)-Br$ +HSPr

                         $\downarrow$

         $Os-(C_5H_{10})-NH-\overset{O}{\overset{\|}{C}}-(CH_2)-S-Pr$
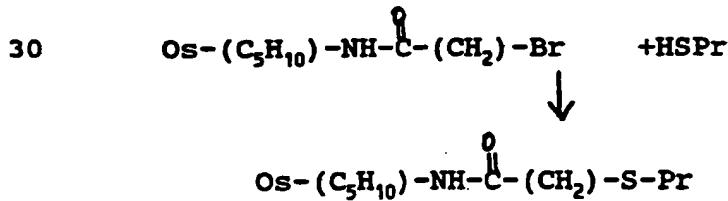
35 wherein an oligosaccharide activated with N-bromoacetyl-

aminopentyl group is allowed to react with a protein that
has been suitably prepared, or

$$Os-NH-\overset{O}{\overset{\|}{C}}-CH_2-CH_2-\overset{O}{\overset{\|}{C}}-N_3 \quad + \quad H_2N-Pr$$

$$\downarrow$$

$$Os-NH-\overset{O}{\overset{\|}{C}}-CH_2-CH_2-\overset{O}{\overset{\|}{C}}-NH-Pr$$
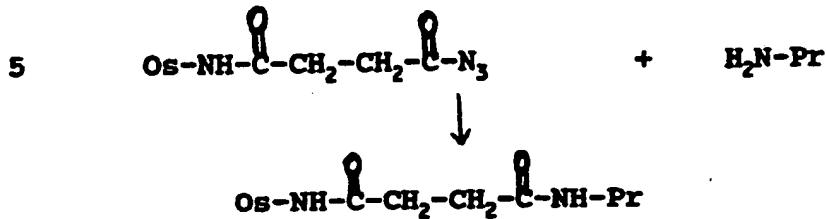
wherein an oligosaccharide activated with an acylazide group
is allowed to react with a protein that has been suitably
prepared. The excess activated oligosaccharide derivative
may be removed by standard methods such as dialysis and gel
filtration.

   F.   Purification of the Oligosaccharide-Protein Conjugate
        The protein-oligosaccharide conjugate may be purified
by affinity lectin chromatography. For example,
Concanavalin A is a lectin which has high specificity for
high-mannose type oligosaccharide. In a typical example of
purification using Con A lectin, the mixture of unreacted
native protein and high-mannose oligosaccharide-protein
conjugate is applied onto a Con A lectin column which is
equilibrated at a temperature of 4° to 25°C with the
appropriate buffer. The unreacted native protein is
collected in the void volume of the lectin column while the
neoglycoprotein is eluted with 0.5M methyl mannoside. The
excess methyl mannoside may be removed by dialysis, by gel
filtration or by ultrafiltration through an Amicon system.
        High performance liquid chromatography (HPLC) may also
be used to purify neoglycoprotein from unreacted native
protein. Glycosylation of proteins usually leads to
increased hydrophilicity, which leads to a shorter retention
time on a reverse-phase HPLC column. Ion exchange HPLC may
also be applied towards the purification of proteins
modified with ionic oligosaccharides, such as those
containing sialic acids.

ficin, and carboxypeptidases. The peptide fragments thus
obtained may be analyzed by HPLC methods and those fragments
containing the radiolabeled cysteine are collected. The
amino acid sequence of these fragments may be determined by
5   standard procedures. A comparison of the amino acid
sequence of these fragments with those of the native protein
may yield information on the exact location of the labeled
cysteine residue.

In accordance with the invention, formation of
10 neoglycoprotein conjugates from oligosaccharides and
therapeutically valuable proteins can provide proteins with
improved biological and physiochemical properties. The
neoglycoprotein conjugates may have reduced tendencies
toward aggregation and higher solubilities in aqueous
15 medium. For example, the high mannose oligosaccharide-
modified G-CSF has a shorter retention time on a reverse
phase HPLC column, which suggests higher hydrophilicity.
The addition of an oligosaccharide moiety to a
therapeutically useful protein may also enhance its
20 stability towards denaturation and proteolytic degradation.
The neoglycloproteins may also be used for improved drug
delivery. Due to the specific interaction between
oligosaccharides and cell surface receptors,
neoglycoproteins may be delivered to specific tissues of the
25 body. For example, high mannose oligosaccharide-modified
proteins may be targeted specifically to macrophages where
cell surface receptors for high mannose oligosaccharides are
located. In addition, complex-type oligosaccharide-modified
proteins may be targeted specifically to hepatocytes.
30   Examples
Example 1: Preparation of the Oxazoline Derivative of 2-
Acetamido-1,3,4,6-tetraacetyl-2-deoxy-α-D-glucopyranose
(GlcNAc peracetate).

In an oven-dried flask, under a nitrogen atmosphere 2-
35 acetamido-1,3,4,6-tetraacetyl-2-deoxy-α-D-glucopyranose (50
mg, 0.128 mmol) was dissolved in 2 ml of anhydrous
dichloroethane (Aldrich). Addition of bromotrimethylsilane

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(Aldrich, 88.5 μl, 0.64 mmol), boron trifluoride etherate
(Aldrich, 79 μl, 0.64 mmol) was made via syringe. The
mixture was stirred at room temperature for 6 hr and then
diluted with methylene chloride (10 ml). The reaction
5 mixture was washed with sat. $NaHCO_3$, H_2O and sat. NaCl
solution. The organic layer was dried (Na_2SO_4) and
concentrated in vacuo. The crude material was purified by
flash chromatography on silica gel (Kieselgel 10: 230-400
mesh) using 40:1 (v/v) chloroform/methanol as eluant to give
10 48.5 mg (97%) of the desired oxazoline as a colorless oil.
The oxazoline will be ideally characterized by the
following: Rf = 0.50, silica gel 60F254(Merck), CH_3Cl_3-MeOH
(10:1); 400 MHZ ^{1}H NMR ($CDCl_3$) ppm 5.98(d) 1 H, 5.28(s) 1 H,
4.95(d) 1 H, 4.17(m) 3 H, 3.60(m) 1 H, 2.12(m) 12 H.
15 Example 2: Preparation of the Oxazoline Derivative of
Chitobiose (GlcNAc β-1→4GlcNAc) Peracetate.

In an oven-dried flask under N_2 atmosphere chitobiose
(GlcNAc β-1→4GlcNAc) (Sigma, 135 mg, 0.2 mmol) was dissolved
in 4 ml of anhydrous 1,2-dichloroethane (Aldrich). Addition
20 of bromotrimethyl silane (Aldrich, 124 μl, 0.9 mmol), boron
trifluoride etherate (Aldrich, 111 μl, 0.9 mmol) and 2,4,6-
collidine (Aldrich, 120 μl, 0.9 mmol) was made via syringe.
The mixture was allowed to stir at 20°C overnight.
Methylene chloride (15 ml) was added to the reaction mixture
25 and the reaction mixture was washed with sat. $NaHCO_3$
solution, H_2O and sat. NaCl solution. The organic layer was
dried (Na_2SO_4) and concentrated in vacuo. The crude material
was purified by flash chromatography (2:1 EtOAc/hexane; 10:1
$CHCl_3$/MeOH) on silica gel (230-400 mesh, EM Science) to give
30 the desired product as an amber oil (70 mg, 57%). The
chitobiose oxazoline product will be ideally characterized
by the following: Rf = 0.55, silica gel 60F254 (Merck),
$CHCl_3$-MeOH (10:1); 400 MHZ ^{1}H NMR ($CDCl_3$) ppm 5.86(m) 2 H,
5.20(t) 1 H, 5.80(t) 1 H, 4.72(d) 1 H, 4.32(m) 2 H, 4.14(m)
35 3 H, 3.92(m) 1 H, 3.75(m) 1 H, 3.50(d) 1 H, 3.42(m) 1 H,
2.20-1.95(m) 18 H, 1.69(s) 3 H.

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Example 3: Process for the Preparation of the Oxazoline
from a Man$_n$GlcNAc Oligosaccharide.

In an oven-dried flask, under a N$_2$ atmosphere, was
dissolved 20 mg (0.01 mmol) of the Man$_n$GlcNAc peracetate
5   (where n is an integer in the range from and including 5
through 8) in 1 ml of anhydrous 1,2-dichloroethane.
Addition of bromotrimethylsilane (Aldrich, 40 μl, 0.29
mmol), boron trifluoride etherate (Aldrich, 40 μl, 0.30
mmol) and 2,4,6-collidine (Aldrich, 45 μl, 0.3 mmol) was
10 made via syringe. The mixture was allowed to stir at 20°C
overnight. Methylene chloride (10 ml) was added to the
reaction mixture and the organic layer was washed with sat.
NaHCO$_3$ solution, H$_2$O and sat. NaCl solution. The organic
layer was dried (Na$_2$SO$_4$) and concentrated in vacuo. The
15 crude material was purified by flash chromatography (40:1
CHCl$_3$-MeOH) on silica gel (230-400 mesh, EM Science) to give
the oligosaccharide peracetate oxazoline as a white solid
(20 mg, 95%). The oligosaccharide oxazoline will be ideally
characterized by its unique NMR resonance for the anomeric
20 proton: 400 MHZ ^{1}H NMR (CDCl$_3$) ppm 5.98 (d,J = 8.5 Hz) 1 H.
Example 4: Preparation of 7S-Oligosaccharide - Bovine Serum
Albumin Conjugate.
A.   Synthesis of Activated 7S-Oligosaccharide Derivative
      (Fig. 3):
25   The 7S-oligosaccharide mixture 1 (Fig. 4), released
from 7S globulin fractions of soybean meal by endonuclease
H, is comprised of a mixture of high mannose type
oligosaccharides with the general structure represented by
Man$_4$-9-GlcNAc (Fig. 4). The 7S-oligosaccharide mixture 1
30 (Fig. 4) (254 mg) is treated with acetic anhydride in
pyridine, at 22 - 25°C, under a nitrogen atmosphere, for 16
hrs. The 7S-oligosaccharide peracetate 2 (Fig. 4) forms a
precipitate upon addition of ice. The peracetate is
collected and dissolved in a minimum amount of chloroform.
35 It is then further purified by silica gel column
chromatography (Kieselgel 60, 230-400 mesh, EM Science).
The column is eluted first with 2:1 (v/v) ethyl

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acetate/hexane to remove the excess pyridine, then with 10:1
(v/v) chloroform/methanol, to give the desired 7S-
oligosaccharide-peracetate 2 as a white solid (331 mg,
Rf = 0.52, 10:1 v/v CHCl₃/MeOH).

5   To a solution of the 7S-oligosaccharide-peracetate 2 in
1,2-dichloroethane is added 5 to 10 molar equivalents of
boron trifluoride etherate, bromotrimethylsilane and
collidine. The reaction mixture is kept at 22 - 25°C, with
stirring, under a nitrogen atmosphere for 16 hrs. Analysis
10 of the reaction mixture by thin layer chromatography (10:1
chloroform/methanol, v/v) indicates the formation of a new
species (Rf = 0.60, 10:1 v/v CHCl₃/MeOH). Dichloromethane
is added to the reaction mixture, which is washed with
saturated aqueous sodium bicarbonate and brine; dried with
15 anhydrous sodium sulfate and evaporated under reduced
pressure to dryness. Purification by silica gel
chromatography (Kieselgel 60, 230-400 mesh, EM science,
eluted first with 2:1 v/v ethyl acetate/hexane, then with
40:1 v/v chloroform/methanol) affords the 7S-oligosaccharide
20 oxazoline-peracetate 3 (Fig. 4) as a white solid (245 mg).

        The oligosaccharide oxazoline-peracetate 3 so obtained
is dissolved in dry 1,2-dichloroethane and treated with five
to ten molar equivalents of 5-(N-trifluoroacetyl)amino-1-
pentanol and p-toluenesulfonic acid. The reaction mixture
25 is kept at ambient temperature (22-25°C), under a nitrogen
atmosphere for 16-48 hrs. Analysis by thin layer
chromatography (10:1 v/v chloroform/methanol) shows the
formation of a new species 4 (Fig. 4) with lower Rf than the
starting oxazoline. The reaction mixture is evaporated
30 under reduced pressure to dryness and is used in the next
step without purification.

        The O-acetates and N-trifluoroacetates on the (N-
trifluoroacetyl)5-aminopentyl-7S-oligosaccharide-peracetate
4 are removed by treatment with 0.2M sodium methoxide in
35 methanol for 16 hrs at ambient temperature. At the end of
the said time period, the excess methoxide is neutralized by
bubbling carbon dioxide through the reaction mixture for 30

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min. The methanol solvent is evaporated under reduced
pressure and the resulting solid dissolved in distilled
water. The aqueous solution of 5-aminopentyl-
oligosaccharide 5 (Figs. 4 and 5) thus obtained is desalted
5 using an Amicon ultrafiltration system (YC05 membrane,
molecular weight cutoff: 500). The aqueous solution
retained in the ultrafiltration unit is lyophilized to a
powder. The powder so obtained is further purified on a
Biogel-P2 gel filtration column equilibrated with 50 mM
10 ammonium bicarbonate solution. Fractions containing the
desired 5-aminopentyl-oligosaccharide 5 are combined and
lyophilized to yield a white fluffy powder (52 mg). The 400
MHZ ^{1}H NMR of the white powder confirms the presence of a β-
5-amino-pentyl glycoside: 4.42 ppm (d, J = 8 Hz, 1H,
15 anomeric H), 2.90 ppm (t, J = 7 Hz, 2H, C$\underline{H}_2$NH), 1.95 ppm (s,
3H, -NCOC$\underline{H}_3$), 1.57 ppm (t, J = 7 Hz, 2H, -C$\underline{H}_2$-CH$_2$-NH$_2$), 1.50
ppm and 1.40 ppm (m, 2H and 2H, -O-CH$_2$-C$\underline{H}_2$-C$\underline{H}_2$-CH$_2$-CH$_2$-NH$_2$).

To a suspension of 5-aminopentyl-7S-oligosaccharide 5
(Fig. 5) (8 mg) in dry dimethyformamide is added a solution
20 of N-hydroxysuccinimidyl ester of bromoacetic acid (0.06 M,
200 μl). The reaction is allowed to proceed at ambient
temperature for 4 hrs, then kept at -10°C for 16 hrs. The
solvent is evaporated on a nitrogen evaporator. The N-
bromoacetyl-5-aminopentyl-7S-oligosaccharide 6 (Fig. 5) is
25 precipitated by the addition of acetone. The precipitate is
washed twice with acetone and the solvent removed by
centrifugation. The white precipitate isolated is further
purified over a Biogel P2 gel filtration column equilibrated
with distilled water. All fractions containing
30 carbohydrates are combined and lyophilized to give 5.0 mg of
a white powder. ^{1}H NMR (400 MHz) shows a downfield shift of
0.25 ppm for the methylene group next to the amine,
confirming that the pendant amino group has been
bromoacetylated: 4.42 ppm (d, J = 8 Hz, anomeric H), 3.15
35 ppm (m, C$\underline{H}_2$NHC(O)CH$_2$Br), 1.97 ppm (s, NHC(O)C$\underline{H}_3$), 1.36 and
1.4 - 1.6 ppm (m, 6H total, -O-CH$_2$-C$\underline{H}_2$-C$\underline{H}_2$-C$\underline{H}_2$-CH$_2$-NH).

B.   Conjugation of Activated 7S-Oligosaccharide Derivative
      with Bovine Serum Albumin (BSA).

      The 7S-oligosaccharide was covalently attached to an
exposed, accessible cysteine on the protein, namely Cys-34
5 of bovine serum albumin. To the N-bromoacetyl-5-
aminopentyl-7S-oligosaccharide derivative obtained as
described above is added a solution containing 0.5 mg/ml of
bovine serum albumin in 0.05M sodium phosphate, 1mM EDTA, 1%
glycerol, pH 7.5. The molar ratio of the activated
10 oligosaccharide derivative to the protein is preferred to be
20:1 or greater. The mixture so obtained is kept in the
dark, under a nitrogen atmosphere for 16 hrs. At the end of
the said time period, the mixture is dialyzed against
several changes of water.

15 C.   Analysis of the 7S-Oligosaccharide - BSA Conjugate

      The reaction mixture so obtained was analyzed by SDS-
polyacrylamide gel electrophoresis. Concanavalin A lectin
blot of the gel shows high mannose oligosaccharide
associated only with new oligosaccharide-protein conjugate.

20 D.   Purification of the 7S-Oligosaccharide - BSA Conjugate.

      An aliquot of the reaction mixture (823 μg of BSA) was
dialyzed exhaustively into 0.01M sodium phosphate and 0.5M
sodium chloride, pH 6.8. The dialysate was passed slowly
through a Concanavalin A lectin column (0.5 ml) equilibrated
25 with 0.01M sodium phosphate, 0.5M sodium chloride, 1mM
$MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, pH 6.8. Fractions
containing unreacted BSA, which did not bind to the lectin
column, were assayed by the Bradford assay and pooled (371
μg). The 7S-oligosaccharide-BSA conjugate, which bound to
30 the lectin column, was eluted with 0.01M sodium phosphate,
0.5M sodium chloride, 0.5M methyl mannoside, pH 6.8.
Fractions collected were assayed according to Bradford and
fractions containing the neoglycoprotein were combined (113
μg).

Example 5

A.   **Preparation of 7S-Oligosaccharide-γ-Interferon Conjugate.**

This example describes the covalent attachment of 7S-
5   oligosaccharide to a cysteine on murine-γ-interferon. This
cysteine is exposed by reductive cleavage of an
intermolecular disulfide bridge. The murine γ-interferon is
obtained from Genentech Co., California. To 1 mg (0.07
μmol) of γ-interferon in 1 ml of 0.1M sodium phosphate, 0.5M
10   sodium chloride pH 7.8 is added β-mercaptoethanol (12 μl,
0.17 mmole). The mixture so obtained is incubated at
ambient temperature (22 - 25°C), in the absence of light,
under a nitrogen atmosphere for 3 hrs. The reduced murine
γ-interferon is purified on a Biogel-P6 column equilibrated
15   with 0.05M sodium phosphate, 1 mM EDTA, 5% glycerol pH 6.8.
The protein, eluted in the void volume, is added to
bromoacetylated-5-aminopentyl-7S-oligosaccharide derivative
(2.4 mg, 1.5 μmol). The mixture so obtained is incubated at
ambient temperature (22 - 25°C), in the absence of light,
20   under a nitrogen atmosphere for 16 hrs.

B.   **Analysis of the Reaction Mixture of γ-Interferon and Activated 7S-Oligosaccharide Derivative**

Analysis of the reaction mixture so obtained by SDS-
PAGE shows the formation of a new protein species which is
25   approximately 2 kDa larger than native γ-interferon.
Concanavalin A lectin blot also shows the presence of high
mannose oligosaccharide associated with this new protein
species.

C.   **Purification of 7S-Oligosaccharide-γ-Interferon**
30   **Conjugate.**

The excess bromoacetylated-5-aminopentyl-7S-
oligosaccharide derivative is removed from the reaction
mixture by passing it through a Biogel-P6 gel filtration
column equilibrated with Concanavalin A buffer (0.5M sodium
35   chloride, 0.01M sodium phosphate, 1 mM calcium (II)
chloride, 1 mm magnesium (II) chloride, 1 mM manganese (II)
chloride, pH 6.8). The mixture of modified and unmodified

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γ-interferon thus obtained is adsorbed on a concanavalin A
lectin column. The unmodified γ-interferon is eluted in the
void volume whereas the modified γ-interferon is eluted by
0.5 M methyl mannoside in Con A buffer. The protein
5 concentrations are estimated using Bradford assay.

Example 6:

A.   Preparation of 7S-Oligosaccharide - Granulocyte -
     Colony - Stimulating Factor Conjugate.

     This example describes the covalent attachment of 7S-
10 oligosaccharide to an inaccessible cysteine on a protein,
for example, Cys-17 of granulocyte-colony stimulating
factor. Recombinant granulocyte-colony stimulating factor
(rG-CSF), isolated from Chinese hamster ovary cells, is
obtained from Chugai Pharmaceutical Co., Japan. A solution
15 of rG-CSF (13 nmole/ml, 250 μg/ml) in 4 ml of 0.01M sodium
phosphate, 0.1M sodium chloride, pH 7.9 is added under a
nitrogen atmosphere to a twenty-five fold molar excess of N-
bromoacetyl-5-aminopentyl-7S-oligosaccharide. Solid urea is
added to this mixture so that the final concentration of the
20 denaturant is 6M. The reaction is allowed to proceed in the
dark, at ambient temperature (22-25°C) for 1.5 hrs. The
reaction mixture so obtained is dialyzed against 0.01M
sodium phosphate, 0.1M sodium chloride, 3M urea, pH 7.6 so
as to renature the protein.

25 B.   Analysis of 7S-Oligosaccharide - Recombinant
     Granulocyte - Colony Stimulating Factor Conjugate.

     SDS-Gel electrophoresis of the reaction mixture by non-
reducing, 10 to 20% polyacrylamide gel indicates the
formation of a new protein species with a molecular weight
30 which is approximately 2 kDa larger than the native rG-CSF.
A concanavalin A lectin blot verifies that high mannose
oligosaccharide is only associated with this new protein
species. Analysis of the reaction mixture by reverse-phase
HPLC (Waters deltapak, 5μ particle size, 300 Å pore size,
35 3.9mm x 150 mm, linear gradient of 37% to 43% isopropanol in
0.025M sodium phosphate, pH 6.5, flow rate 1 ml/min.) also

shows that 34% of the rG-CSF has been labelled with the N-
bromoacetyl-5-aminopentyl-7S-oligosaccharide derivative.

C.   **Purification of 7S-Oligosaccharide-G-CSF Conjugate.**

Purification of the 7S-oligosaccharide-G-CSF conjugate

5   is effected by using reverse-phase HPLC. The dialysate is
injected onto a C18 column (Waters deltapak, 5μ particle
size, 300 Å pore size, 3.9mm x 150mm), equilibrated with a
buffer consisting of 37:63 isopropanol (IPA)/0.025 M sodium
phosphate, pH 6.5. The glycoprotein conjugate is separated

10   from the unmodified protein using a linear gradient of 37%
IPA to 43% IPA (flow rate 1 ml/min) as the eluant. The
glycoprotein conjugate has a shorter retention time than the
unmodified protein.

What is claimed is:

1.   An oligosaccharide-spacer conjugate, formed from
an oligosaccharide having a reducing GlcNAc terminus that
has been reacted to form an oxazoline, defining a location
5 to which is coupled a bifunctional spacer.

2.   A conjugate according to claim 1, wherein the
bifunctional spacer is coupled by a β-O-glycosidic bond at
the location.

3.   A conjugate according to claim 2, wherein the
10 oligosaccharide includes a compound of the structure of the
formula: $Man_nGlcNAc$, where n is an integer in the range from
and including 3 through 9.

4.   A conjugate according to claim 2, wherein the
oligosaccharide is a high mannose-type oligosaccharide of
15 the formula: $Man_nGlcNAcGlcNAc$, where n is an integer in the
range from and including 3 through 9.

5.   A conjugate according to claim 2, wherein the
oligosaccharide is a complex-type oligosaccharide.

6.   A conjugate according to claim 5, wherein the
20 oligosaccharide is desialylated.

7.   An oligosaccharide-protein conjugate with an
oligosaccharide having a β-O-glycosidic bond to a
bifunctional spacer coupled to a protein.

8.   A conjugate according to claim 7, wherein the
25 bifunctional spacer has the formula: $-O-(CH_2)_5-NH-\overset{\overset{O}{\parallel}}{C}-CH_2-$.

9.   A conjugate according to claim 7, wherein the
oligosaccharide includes a structure of the formula
$Man_n-GlcNAc$ and n is an integer in the range from and
including 3 through 9.

30   10.   A conjugate according to claim 7, wherein the
protein is serum albumin.

11.   A conjugate according to claim 7, wherein the
protein is granulocyte-colony stimulating factor.

12.   A conjugate according to claim 7, wherein the
35 protein is γ-interferon.

13.   A conjugate according to claim 7, wherein the
oligosaccharide includes a structure of the formula

Man_n-GlcNAc, n being an integer in the range from and
including 3 through 9, the protein is serum albumin, and the
bifunctional spacer may be represented by
$-O-(CH_2)_5-NH-\overset{O}{\overset{\|}{C}}-CH_2-$.

5   14.   A conjugate, according to claim 7, wherein the
oligosaccharide includes a structure of the formula Man_n-
GlcNAc, n being an integer in the range from and including 3
through 9, the protein is granulocyte-colony stimulating
factor, and the bifunctional spacer has the formula:
10 $-O-(CH_2)_5-NH-\overset{O}{\overset{\|}{C}}-CH_2-$.

        15.   A conjugate according to claim 7, wherein the
oligosaccharide includes a structure of the formula
Man_n-GlcNAc, n being an integer in the range from and
including 3 through 9, the protein is γ-interferon, and the
15 bifunctional spacer has the formula $-O-(CH_2)_5-NH-\overset{O}{\overset{\|}{C}}-CH_2-$.

        16.   A conjugate having the formula: Os-A-B-C,
wherein:
        Os is the oligosaccharide with a terminal GlcNAc;
        A is β-O- or β-N(H)-$\overset{O}{\overset{\|}{C}}$-
20   B is a spacer; and
        C is an electrophilic group.
        17.   A conjugate according to claim 16, wherein the
spacer B has the formula $-(CH_2)_nX-Y-(CH_2)_n-Z$ wherein:
        n is an integer in the range from and including 0
25   through 10;
        X is $\overset{O}{\overset{\|}{C}}$, $\overset{NH}{\overset{\|}{C}}$, $(CH_2)_n$, or N-R and R = H, or CH_3;
        Y is $\overset{O}{\overset{\|}{C}}$, $\overset{NH}{\overset{\|}{C}}$, $(CH_2)_n$, N-R or S; and
        Z is N-R or $(CH_2)_n$.

18. A conjugate according to claim 17, wherein C is an electrophilic group having the formula:

$-\overset{O}{\overset{\|}{C}}$-CH$_2$-halide, where halide is Cl, Br, or I; or
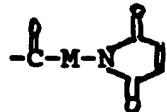
$-\overset{O}{\overset{\|}{C}}$-M-N, where M is an aromatic ring or (CH$_2$)$_n$, where n is an integer from and including 1 through 5; or

$-\overset{O}{\overset{\|}{C}}$-N$_3$, $-\overset{O}{\overset{\|}{C}}$-O-N, or -C-N
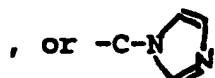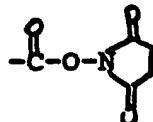
19. A conjugate according to claim 18, wherein the oligosaccharide includes a structure of the formula: Man$_n$GlcNAc, where n is an integer in the range from and including 3 through 9.

20. A conjugate according to claim 18, wherein the oligosaccharide includes a structure of the formula: Man$_n$GlcNAcGlcNAc, where n is an integer in the range from and including 3 through 9.

21. A conjugate according to claim 18, wherein the oligosaccharide is a complex-type oligosaccharide.

22. A conjugate according to claim 21, wherein the oligosaccharide is desialylated.

23. A conjugate between an oligosaccharide having a reducing GlcNAc terminus and a protein having a nucleophile, coupled by a bifunctional spacer having the formula: Os-A-B-C-Nu-Pr, wherein:

Os is the oligosaccharide with a terminal GlcNAc;

A is β-O- or β-N(H)-$\overset{O}{\overset{\|}{C}}$-;

B is a spacer;

C is the product of the reaction between the electrophilic group on the oligosaccharide-spacer conjugate with the nucleophilic group on the protein;

Nu is a nucleophilic group on the protein; and

Pr is the protein.

24. A conjugate according to claim 23, wherein the spacer B has the formula: $-(CH_2)_n-X-Y-(CH_2)_n-Z-$ wherein:

n is an integer in the range from and including 0 through 10;

X is $\overset{O}{\overset{\|}{C}}$, $\overset{NH}{\overset{\|}{C}}$, $(CH_2)_n$, or N-R and R = H or CH_3;

Y is $\overset{O}{\overset{\|}{C}}$, $\overset{NH}{\overset{\|}{C}}$, $(CH_2)_n$, N-R or S; and

Z is N-R or $(CH_2)_n$.

25. A conjugate according to claim 24, wherein the group C has the formula:
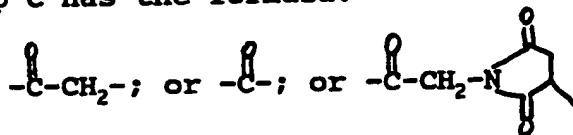
26. A conjugate according to claim 25, wherein the spacer has the formula: $-O-(CH_2)_5-NH-\overset{O}{\overset{\|}{C}}-CH_2-$.

27. A conjugate, according to claim 25, wherein the oligosaccharide includes a structure of the formula Man_n-GlcNAc where n is an integer in the range from and including 3 through 9.

28. A conjugate according to claim 25, wherein the protein is serum albumin.

29. A conjugate according to claim 25, wherein the protein is granulocyte colony-stimulating factor.

30. A conjugate according to claim 25, wherein the protein is γ-interferon.

31. A conjugate according to claim 25, wherein the oligosaccharide includes a structure of the formula Man_n-GlcNAc and n is an integer in the range from and including 3 through 9, the protein is serum albumin and the bifunctional spacer may be represented by $-O-(CH_2)_5-NH-\overset{O}{\overset{\|}{C}}-CH_2-$.

32. A conjugate according to claim 25, wherein the oligosaccharide includes a structure of the formula Man_n-GlcNAc and n is an integer in the range from and including 3 through 9, the protein is granulocyte-colony

stimulating factor, and the bigeneric spacer has the
formula: $-O-(CH_2)_5-NH-\overset{O}{\overset{\|}{C}}-CH_2-$.

33.   A conjugate according to claim 25, wherein the
oligosaccharide includes a structure of the formula
5   Man$_n$-GlcNAc and n is an integer in the range from and
including 3 through 9, the protein is γ-interferon and the
bifunctional spacer has the formula $-O-(CH_2)_5-NH-\overset{O}{\overset{\|}{C}}-CH_2-$.

34.   A method for obtaining an oligosaccharide-spacer
conjugate comprising:
10   (a)   obtaining an oligosaccharide having a reducing
GlcNAc terminus;
          (b)   forming an oxazoline at the GlcNAc terminus;
          (c)   coupling, to the location of the oxazoline, a β-O-
linkage with a bifunctional spacer.
15   35.   A method according to claim 34, wherein the
oligosaccharide obtained in step (a) includes a structure of
the formula: Man$_n$GlcNAc, where n is an integer in the range
from and including 3 through 9.
          36.   A method according to claim 34, wherein the
20   oligosaccharide obtained in step (a) includes a compound of
the formula: Man$_n$GlcNAcGlcNAc, where n is an integer in the
range from and including 3 through 9.
          37.   A method according to claim 34, wherein the
oligosaccharide obtained in step (a) is a complex-type
25   oligosaccharide.
          38.   A method according to claim 34, wherein the
oligosaccharide obtained in step (a) is desialylated.
          39.   A method for obtaining an oligosaccharide-protein
conjugate comprising:
30   (a)   obtaining an oligosaccharide having a reducing
GlcNAc terminus;
          (b)   forming an oxazoline at the GlcNAc terminus;
          (c)   coupling, to the location of the oxazoline, a
bifunctional spacer; and
35   (d)   coupling the oligosaccharide-spacer conjugate to a
cysteine on a protein.

40. A method for obtaining a conjugate according to claim 39, wherein the bifunctional spacer used in step (c) has the formula: $-O-(CH_2)_5-NH-\overset{O}{\overset{\|}{C}}-CH_2-$.

41. A method for obtaining a conjugate according to claim 39, wherein the oligosaccharide obtained in step (a) includes a structure of the formula Man_n-GlcNAc and n is an integer in the range from and including 3 through 9.

42. A method for obtaining a conjugate, according to claim 39, wherein the protein used in step (d) is serum albumin.

43. A method for obtaining a conjugate according to claim 39, wherein the protein used in step (d) is granulocyte-colony stimulating factor.

44. A method for obtaining a conjugate according to claim 39, wherein the protein used in step (d) is γ-interferon.

45. A method for obtaining a conjugate according to claim 39, wherein the oligosaccharide obtained in step (a) includes a compound of the formula Man_n-GlcNAc, n being an integer in the range from and including 3 through 9, the protein used in step (d) is serum albumin and the bifunctional spacer used in step (c) may be represented by $-O-(CH_2)_5-NH-\overset{O}{\overset{\|}{C}}-CH_2-$.

46. A method for obtaining a conjugate, according to claim 39, wherein the oligosaccharide obtained in step (a) includes a compound of the formula Man_n-GlcNAc, n being an integer in the range from and including 3 through 9, the protein used in step (d) is granulocyte-colony stimulating factor, and the bifunctional spacer used in step (c) has the formula: $-O-(CH_2)_5-NH-\overset{O}{\overset{\|}{C}}-CH_2-$.

47. A method for obtaining a conjugate, according to claim 39, wherein the oligosaccharide obtained in step (a) includes a compound of the formula Man_n-GlcNAc, n being an integer in the range from and including 3 though 9, the protein used in step (s) is γ-interferon and the bifunctional spacer used in step (c) has the formula: $-O-(CH_2)_5-NH-\overset{O}{\overset{\|}{C}}-CH_2-$.

48. A process for the production of a peracetyl
oxazoline comprising reacting a peracetyl saccharide with
reagents suitable for generating the formation of one or
·more of the intermediates of the formula:

5
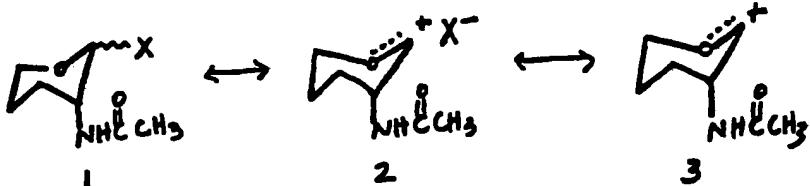
49. A method for the production of a peracetyl
10 oxazoline according to claim 48, wherein the peracetyl
saccharide is selected from mono-, di-, and
oligosaccharides.

50. A method for the production of an oxazoline
according to claim 48, wherein the saccharide may be a
15 pertrichloroacetyl, pertrifluoroacetyl or perbenzoylacetyl
saccharide.

51. A method for the production of a peracetyl
oxazoline according to claim 48, wherein the reagents are
composed of a 1:1 mixture of boron trifluoride etherate and
20 bromotrimethylsilane.

52. A method for the production of a peracetyl
oxazoline according to claim 48, wherein the reagents are
composed of boron trifluoride etherate and
chlorotrimethylsilane.

25   53. A method for the production of a peracetyl
oxazoline according to claim 48, wherein the reagents are
composed of boron trifluoride etherate and
iodotrimethylsilane.

54. A method for the production of a peracetyl
30 oxazoline according to claim 48, wherein the reagents
include the presence of an acid-scavenger.

55. A method according to claim 54, wherein the
reagents include boron trifluoride etherate and
bromotrimethylsilane.

35   56. A method according to claim 54, wherein the
reagents include boron trifluoride etherate and
chlorotrimethylsilane.

57. A method according to claim 54, wherein the acid
scavenger is selected from the group consisting of 2,4,6-
collidine, pyridine, triethylamine, dimethylaminopyridine,
and N,N-diisopropylethylamine.

5   58. A method for the synthesis of a glycopeptide
comprising:

(a) reacting a peracetyl saccharide with a reagent
combination capable of generating a peracetyl oxazoline;

(b) reacting said peracetyl oxazoline with an azide to
10 produce a glycosyl azide;

(c) reducing said glycosyl azide to produce a glycosyl
amine; and

(d) coupling said glycosyl amine with an activated
carboxylic group in an amino acid or peptide.

15   59. A method according to claim 58, wherein the
saccharide is a peracetyl, a pertrichloroacetyl, a
pertrifluoroacetyl or perbenzoyl saccharide.

60. An oligosaccharide-oxazoline comprising a
carbohydrate structure having the formula:

20

wherein R_1, is selected from the group consisting of:

(a) man_n where n is an integer; and
25   (b) a structure having the formula:

30
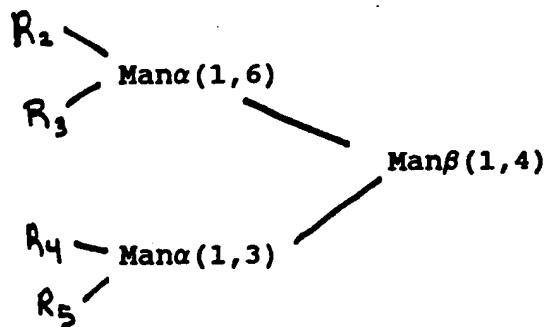
35   wherein R_2, R_3, R_4, and R_5 are selected from the
group consisting of hydrogen and structures containing

one or more coupled molecules selected from the group consisting of hexose and hexosamine.

61. An oligosaccharide oxazoline according to claim 60, wherein the hexose is selected from the group consisting of mannose, galactose, glucose, and fucose, and the hexosamine is selected from the group consisting of N-acetyl glucosamine and N-acetylgalactosamine.

62. An oligosaccharide oxazoline according to claim 60, wherein n is greater than 2.

FIG. I

FIG. 2

$Man\alpha(1\rightarrow3)$
$\quad\searrow$
$\quad\quad Man\beta(1\rightarrow4)GlcNAc$
$\quad\nearrow$
$Man\alpha(1\rightarrow3)$
$\quad\searrow$
$\quad Man\alpha(1\rightarrow6)$
$Man\alpha(1\rightarrow6)$

$Man_5GlcNAc$

$Man\alpha(1\rightarrow2)-Man\alpha(1\rightarrow3)$
$\quad\quad\quad\quad\quad\quad\quad\searrow$
$\quad\quad\quad\quad\quad\quad\quad Man\beta(1\rightarrow4)GlcNAc$
$\quad\quad\quad\quad\quad\quad\nearrow$
$Man\alpha(1\rightarrow3)$
$\quad\searrow$
$\quad Man\alpha(1\rightarrow6)$
$Man\alpha(1\rightarrow6)$

$Man_6GlcNAc$

$Man\alpha(1\rightarrow2)-Man\alpha(1\rightarrow2)-Man\alpha(1\rightarrow3)$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\searrow$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad Man\beta(1\rightarrow4)GlcNAc$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\nearrow$
$Man\alpha(1\rightarrow3)$
$\quad\searrow$
$\quad Man\alpha(1\rightarrow6)$
$Man\alpha(1\rightarrow6)$

$Man_7\,GlcNAc\ isomer$

Man α (1→2) — Man α (1→3)

Man β (1→4) GlcNAc

Man α (1→6)

Man α (1→3)

Man α (1→2) — Man α (1→6)

Man₇ GlcNAc isomer

Man α(1→2) Man α (1→2) Man α (1→3)

Man β (1→4) GlcNAc

Man α (1→3)

Man α (1→6)

Man α (1→2) Man α (1→6)

Man₈ GlcNAc
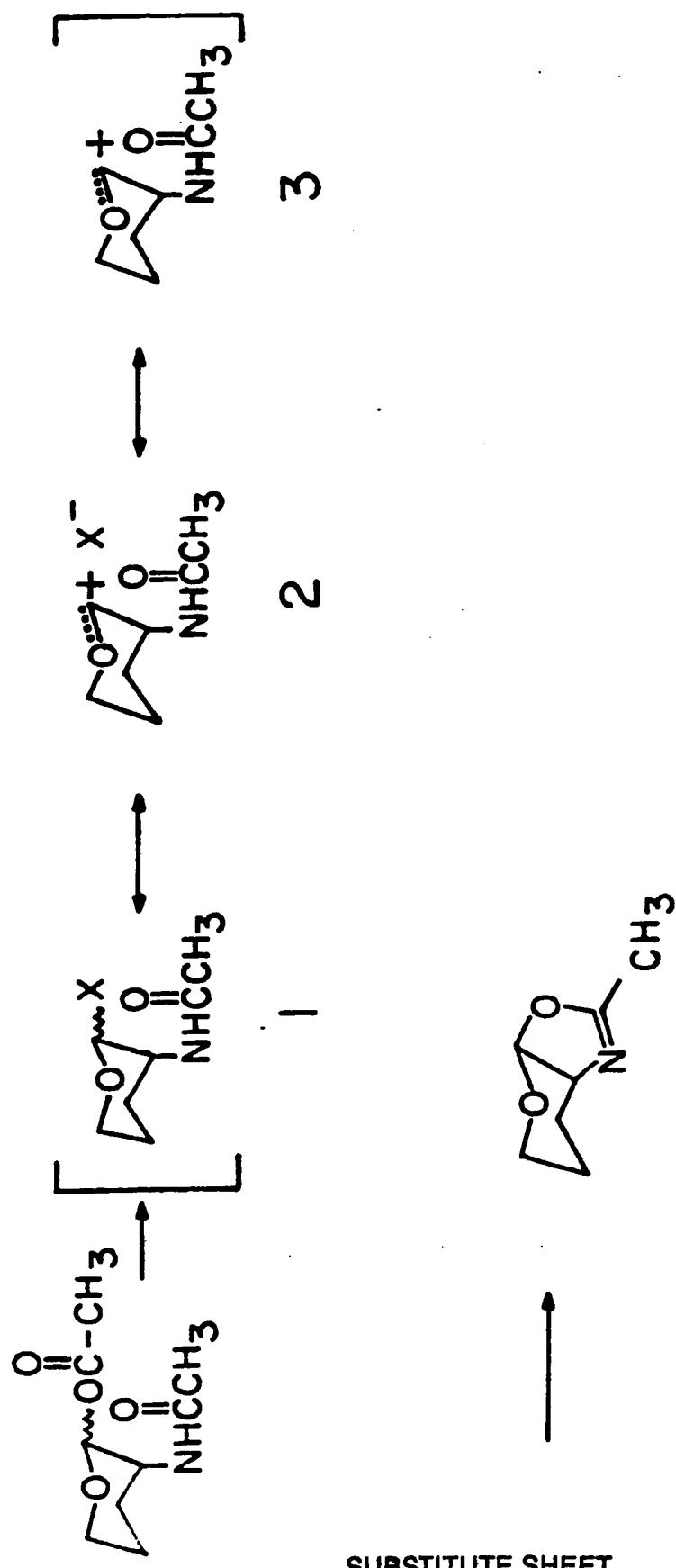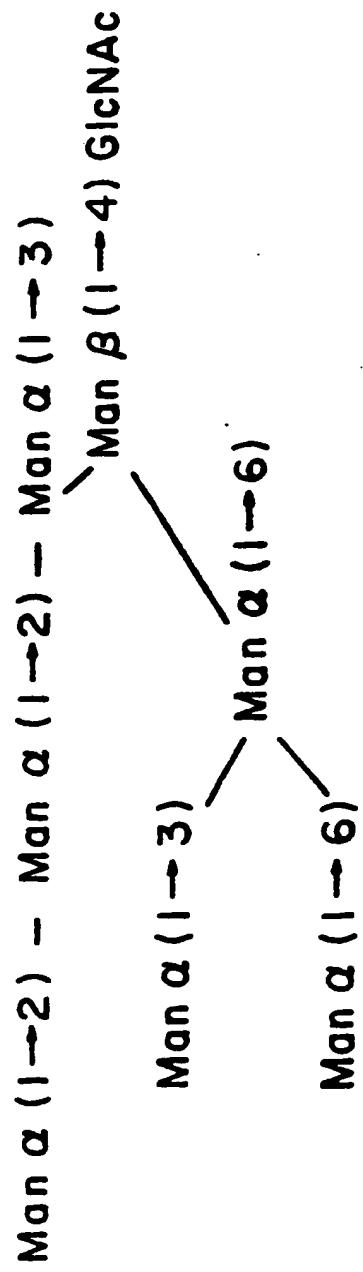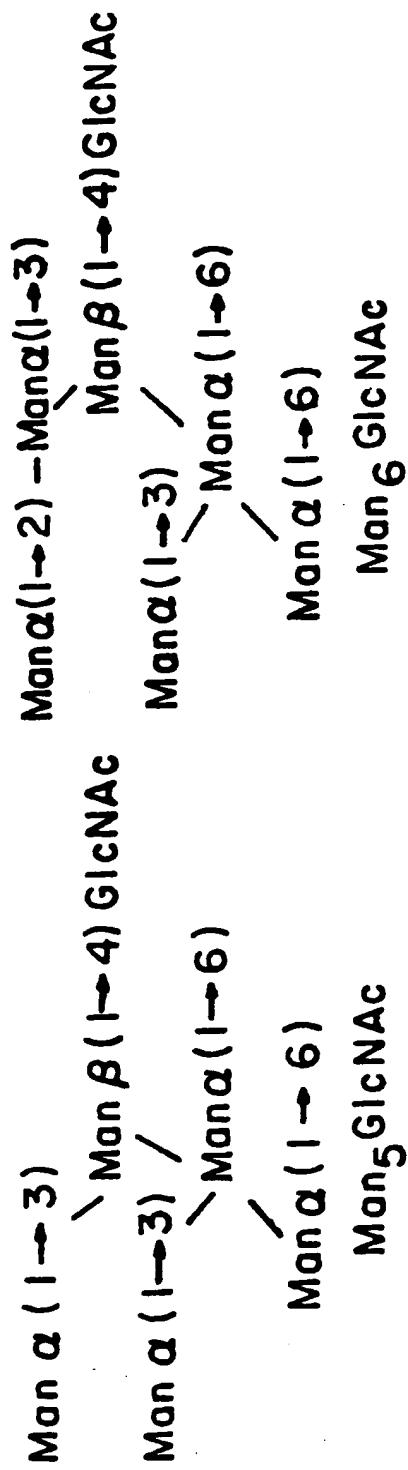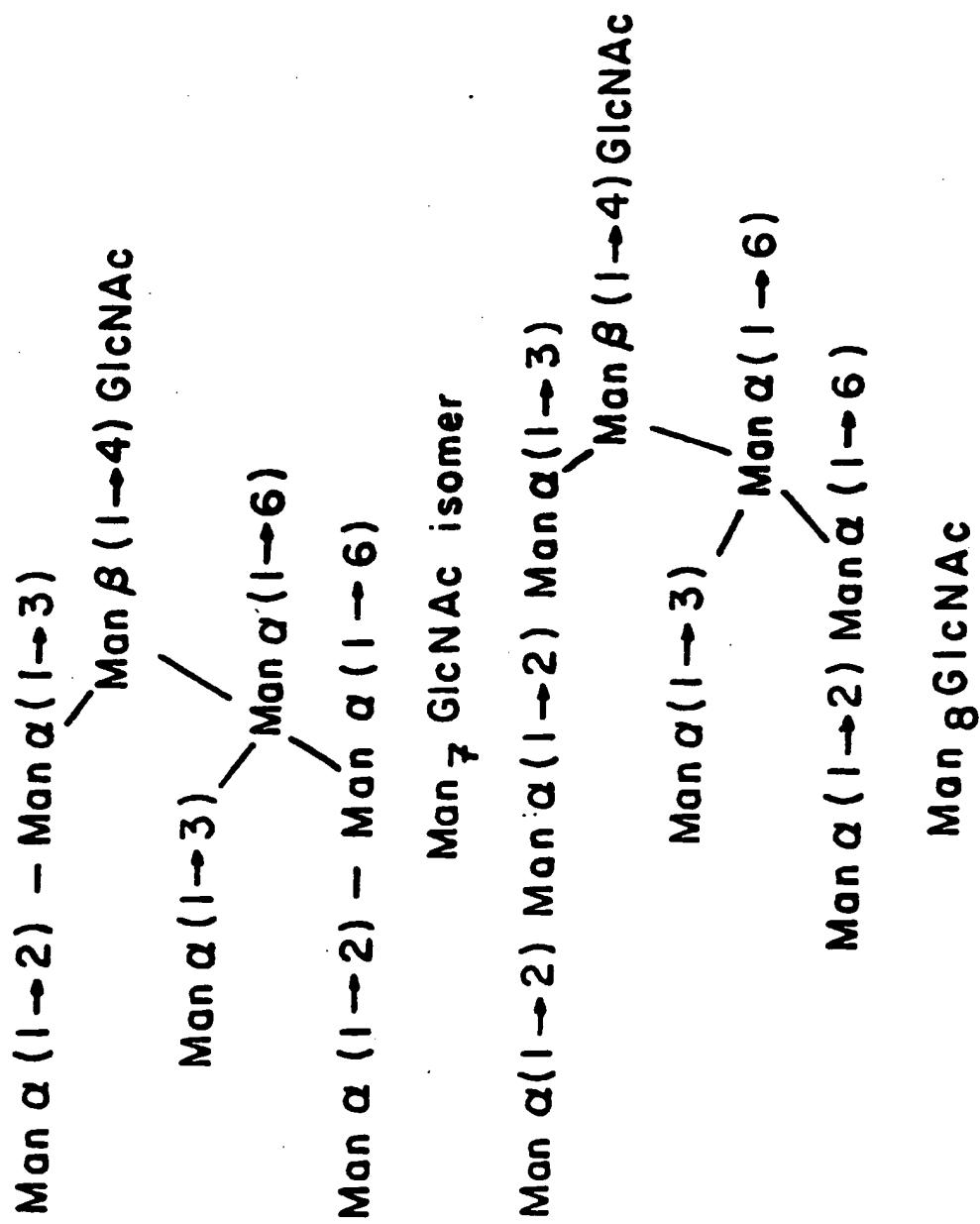
FIG.3

4/5   R = 7S OLIGOSACCHARIDE
R' = PERACETYLATED
7S OLIGOSACCHARIDE

FIG. 4   SUBSTITUTE SHEET

5/5

(4)

$\downarrow$ NaOMe, MeOH

R0 —, HO —, HNAC H, OH, O-(C$_5$H$_{10}$)-NH$_2$

(5)

O=, N-O-, O=, CH$_2$-Br

$\downarrow$

R'O —, HO —, AcNH, OH, O-(C$_5$H$_{10}$)NH-C(=O)-CH$_2$-Br

(6)

Pr = PROTEIN

HS-Pr

$\downarrow$

R'O —, HO —, NHAC, O-(C$_5$H$_{10}$)NH-C(=O)-CH$_2$-S-Pr

FIG. 5